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Identification and quantification of seleno-proteins by 2-DE-SR-XRF in selenium-enriched yeasts

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A comprehensive approach that can identify and quantify selenium (Se) in seleno-proteins in Se-enriched yeast was developed. The Se-containing compounds in Se-enriched yeast were first extracted and then the fraction of Se-containing proteins in the supernatant was analyzed by 2-dimensional electrophoresis (2-DE) and synchrotron radiation X-ray fluorescence (SR-XRF). The detection limit (DL) of SR-XRF analysis for Se quantification in Se-containing proteins after 2-DE separation was calculated to be 0.20 μg g^{-1}, which is suitable for Se quantification in the Se-containing spots present on the 2-D gel. After being scanned by SR-XRF, only spots with a mean Se content exceeding twice the DL of SR-XRF were considered to be seleno-proteins. In this way, a total of 157 Se-containing spots in the gel were visually distinguished. Se contents in all the Se-containing proteins of different molecular weights were quantified. The total Se content on the 2-D gel was calculated to be 126.56 μg g^{-1}, which covered most of the seleno-proteins on the 2-D gel.

Introduction

Selenium (Se) is an essential micronutrient for living organisms but is toxic at high concentration.[1,2] The deficiency of Se can lead to serious health problems, such as Keshan disease and Kashin Beck disease.[3] Various Se-enriched foods and supplements, like Se-enriched rice, yeast, and mushroom, are commercially available. The bioavailability and toxicity of Se are closely correlated with its chemical form.[4,5] Speciation analysis of Se in supplements is necessary and has been carried out by many researchers.[6–9] Se-enriched yeast is an attractive Se supplementary source due to its ability to act as a precursor for selenoprotein synthesis and its high content of selenomethionine (SeMet), which is a highly bioavailable Se form with low toxicity. Significant efforts have been made in the development of analytical methods for the speciation of Se in biological samples in recent years.[10–12]

The separation of Se-containing compounds can be achieved using HPLC, CE or GC, of which HPLC is the most frequently used because of its better reproducibility and higher efficiency.[6,13,14] HPLC combined with ICP-MS is mostly used to analyze Se content and speciation in Se-containing compounds, which can be used to identify Se species at the μg g^{-1} level.[8,15,16] However, HPLC also has its limitations. For example, reversed phase HPLC (RP-HPLC) can only separate compounds with no or little polarity and cannot analyze macromolecular compounds like proteins and polypeptides.

Electrophoresis has a high resolution for the separation of macromolecular compounds. One-dimensional (1D) electrophoresis is a suitable tool for separation of macromolecular compounds like proteins and polypeptides, but cannot separate compounds with a molecular weight difference less than 10%. Two-dimensional (2D) gel electrophoresis, which has higher resolution, better intensity and lower detection limits than 1-D gel electrophoresis, is a perfect approach to analyze Se-containing compounds in organisms.[17,18]

The detection and quantification of metal containing macromolecules in biological samples has received increasing attention. In comparison with analysis of liquid samples by a chromatographic technique, the direct analysis of metalloproteins on solid samples, like electrophoresis gels, requires specific analytical methods able to detect metals *in situ*. Many methods like laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), particle induced X-ray emission (PIXE), and synchrotron radiation X-ray fluorescence (SR-XRF), as well as X-ray absorption spectroscopy (XAS), which can provide the local structural environment of metal ions, are suitable to apply to the analysis of metals in biomacromolecules on solid samples.[19–22] Therefore, SR-micro (μ)-XRF which has a lower detection limit than PIXE, smaller spatial resolution and causes less damage to the samples than LA-ICP-MS should be a potentially promising technique for metal identification after

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electrophoresis separation. Gao *et al.* studied the metalloproteins in human liver cytosol by SR-XRF combined with gel filtration chromatography and isoelectric focusing separation, and 2 Zn-containing bands, 11 Fe-containing bands and some Cu-containing bands with specific pI from 4–7 were successfully detected.[23] Finney L. *et al.*[24,25] have identified a novel periplasmic zinc protein and some other metal-containing proteins through XRF mapping and mass spectrometry after native 2D gel separation, while without *in situ* quantification of the metal/metalloid in specific metal-containing proteins/peptides.

Synchrotron radiation X-ray fluorescence (SR-XRF) is a highly specific and sensitive method for identification, characterization and distribution analysis of metals and nonmetals in biological samples.[26–30] SR-XRF is capable of microscopic analysis and can supply information about 2-D distribution of trace elements.[31] With the advancement of the third generation synchrotron radiation sources, the SR-XRF technique can probe trace elements in biological specimens with high sensitivities (sub-mg kg^{-1}).[30] Hard X-rays can be focused to a submicron, even to nanometer spot size. This makes it possible and ideal to study the spatial distribution and relative concentration of trace elements in specific proteins by SR-XRF after electrophoresis separation.

Se, as a trace element in the majority of cases, plays an important role in biological activities by way of embedding into some critical proteases, like GSH-Px, selenoprotein P, *etc.* The expression of Se-containing proteins as well as Se contents in specific biomolecules may be quite variable between normal and pathological biological tissues. Therefore, identification of Se containing proteins and precise quantification of Se content in specific biomolecules by effective methods are beneficial for the diagnosis and prevention of Se or other metal and metalloid related diseases.[32–34] In the present study, we took Se-enriched yeast as a simple model organism to identify Se-containing proteins and quantify Se contents in these biomolecules by 2DE-SR-XRF. The Se-containing proteins in Se-enriched yeast were extracted first, then separated by 2-DE and scanned by SR-XRF. Using the SR-XRF technique, Se contents in Se-binding proteins can be readily detected and quantified with high sensitivity in 2-DE gels with less damage to the primary separated macromolecules.

Materials and methods

Reagents and standards

SelenoPrecise® yeast (*S. cerevisiae*, Se-enriched yeast) was supplied by Pharma Nord (Denmark). 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), dithioerythritol (DTT), 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Pefabloc®SC), DNase I and RNase A, and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich (St Louis, MO, USA). Pronase E was from Merck (Germany); methanol was from Dikmanpure (USA); heptafluorobutyric acid was from Fluka (Germany). Nitric acid (65%), hydrogen peroxide (30%) and other reagents are all of analytical reagent grade and were purchased from Sinopharm (China). All samples and solutions were prepared with deionized water obtained from Milli-Q (Millipore, UK).

Determination of the total Se by ICP-MS

Yeast and extracts were digested with a mixture of nitric acid and hydrogen peroxide (4 : 1, v/v) at 160 °C for 12 h. The resulting solutions were volatilized at temperatures below 90 °C to a volume of 0.1–0.2 mL, and then diluted with 2% nitric acid up to 2 mL for Se determination by ICP-MS (Thermo Elemental X 7). A collision cell technology (CCT) was employed for avoiding the ^{40}Ar interference. Indium (SpexCertiprep Corp. USA, 10 μg L^{-1}) was used as the internal standard for all sample solutions. The calibrating standards were prepared by diluting the Se standard stock solution (100 mg L^{-1} Quality Control Standard 21, SpexCertiprep Co. USA) with 2% (v/v) nitric acid and then measured in the same manner with the supernatant.[26,33] Deionized water was used as the reagent blank and a certified reference, bovine liver CRM (1577a, NBS, USA) with a certified Se value of 0.56 ± 0.07 μg g^{-1} was used to check the validity of the method.

Extraction of proteins from Se-enriched yeast

Three replicates of 0.4 g Se-enriched yeast were added to 5 mL of extracting buffer which contains 7 M urea, 2 M thiourea, 4% w/v CHAPS, 1% w/v DTT, 2% v/v carrier ampholytes at pH 3–10, 10 mM Pefabloc®sc proteinase inhibitor, 20 μg mL^{-1} DNase I, and 25 μg mL^{-1} RNase A and ultrasonicated for 30 min in an ice bath. The mixture was centrifuged (Hitachi CP 80MX, Japan) at 16 000× *g* for 30 min at 4 °C.[14,35] The supernatants were collected and then stored at −20 °C until analysis. The solid residue was digested with nitric acid and hydrogen peroxide (4 : 1, v/v) for total Se analysis by ICP-MS.

The protein contents in the supernatants were quantified by the Bradford method. The Se content in the supernatants and ultimate residue was measured by ICP-MS after digestion for evaluation of the extracting efficiency and Se recovery.

Separation of Se-containing proteins by 2-D gel electrophoresis

Isoelectric focusing (IF) gel electrophoresis was performed with a Protean II IEF Cell system (Bio-Rad, USA). The protein content of the macromolecular fraction was adjusted to 0.2–0.5 mg mL^{-1} with IPG strip rehydration solution (8 M urea, 4% CHAPS, 65 mM DTT, 0.001% bromophenol blue). Each of the 125 μL protein solutions was loaded to the sample channel. Immobilized pH gradient (IPG) gel strips (7 cm) with a linear pH range (3–10) were soaked in the solution for 12 h. Afterwards, IEF was run at 20 °C and 0.05 mA per strip up to 32 375 V h, in a stepped manner of an initial voltage of 250 V for 30 min, and then 500 V for 30 min, and finally 4000 V for 8 h.[29]

After focusing, the strips were soaked in the equilibration buffer containing 6 M urea, 0.375 M Tris–HCl (pH 8.8), 20% v/v glycerol and 2% w/v SDS, with the addition of 2% w/v DTT for 15 min, followed by the same buffer without DTT but with the 2.5% w/v iodoacetamide (ICA) for 15 min. In the second dimension, the strips were applied to 12% SDS-PAGE gels (20 × 16 × 1 mm)

and electrophoresis was performed in a Protean II Xi (Bio-Rad, USA) apparatus. The electrophoresis was run at 5 mA for 1 h, then at 20 mA until the dye front reached the edge of the gel. The separated proteins were stained by using Simply-Blue™SafeStain and Coomassie R-250 (Sigma) and dried at 80 °C with a vacuum gel drying system (583 type, Bio-Rad, USA).[29]

Analysis of Se-containing proteins in the gels by SR-XRF

Se-containing protein imaging in the unstained 2-DE gel was carried out at BL-4A in Photon Factory, High Energy Accelerator Research Organization (KEK), Japan. The storage ring runs at an energy of 2.5 GeV with a current intensity of 350–450 mA. A monochromatic SR with a photon energy of 13.5 keV and a spot size of 1 × 1 mm^2 was used to excite the samples. The dried gel was moved along the horizontal and vertical direction at an interval of 1 mm for each step. The XRF signals were collected with a Si (Li) detector (PGT Inc. LS 30143-DS) up to 10 s for each point. The fluorescence intensity of Se, Fe, Cu, Zn, Mn and Compton scattering was recorded and analyzed by using 6 SCAs (single channel analyzers, Ortec 550), respectively. The calibration curve was obtained by preparing 0 (control gel), 0.5, 1, 5 and 10 mg L^{-1} Se-containing gels, each of which was prepared by adding a certain amount of Se to 5.5 mL 12% SDS-PAGE gel (8.6 × 6.4 × 1 mm). All the standard gels were dried at 80 °C with a vacuum gel drying system (Model 583, Bio-Rad, USA), then they were analyzed according to the same procedure as SR-XRF analysis of the 2-DE gel of the Se-containing yeast sample. The element signal intensity of each point in the Se-containing gel was corrected by subtracting the signal intensity of the control gel (without Se addition).

In order to correct the effect of the SR beam flux variation on the signal intensity, the intensity of each pixel (*i.e.*, element) in the gel was calibrated by Compton scattering, and then used for calculating the Se content according to the calibration curve. In addition, amounts of Se-containing spots present on the gel are bigger than 1 × 1 mm^2 (the beam spot size) in fact, in other words, each protein spot may be covered by several beam spots. To avoid the determination biases due to unmatched size between the protein spots and the beam spots as well as inhomogeneous distribution of Se within the protein, we use the sum of the values of several beam spots which can roughly cover the protein spot to obtain the Se content in each protein spot.

Results and discussion

Extraction of Se-containing proteins from Se-enriched yeast

To evaluate the extract efficiency of the sample preparation procedure, Se contents in the supernatant and residue were measured by ICP-MS. In a linear range of 0–100 μg L^{-1}, the regression coefficient (R^2) is 0.9997. The Se content in the CRM (1577a, NBS, USA) determined is 0.55 ± 0.05 μg g^{-1}, which corresponds to a recovery of 98.2%.

The contents of Se in each fraction obtained, along with the Se recovery, are listed in Table 1. The extracted Se in the supernatant was about 376.5 ± 4.3 μg g^{-1}, with a recovery of about 30%. The low Se recovery of Se-enriched yeast may be ascribed to the insufficient sonication time for this distinct specimen.

Detection limits for Se quantification in Se-containing spots by SR-XRF

The calibration curve for Se quantification using SR-XRF is shown in Fig. 1. The detection limit (DL) in SR-XRF analysis for Se quantification in the Se-containing proteins after 2-DE separation was calculated according to the following equation:[36,37]

$$\mathrm{DL} = \frac{3\sqrt{N_\mathrm{b}}}{N_i} C_i$$

where DL is the detection limit of element i; N_b is the background count of the sample with no element i; N_i is the net count of element i in a standard sample; and C_i is the concentration of element i in the standard sample with element i. From the equation, the decrease of the background count and increase of the net count, as well as the increasing counting time, are all beneficial for improving the sensitivity and decreasing the DL for element i measurement by this technique. In the present study, due to the low background count value, enough counting time (10 s) for each point, the high luminous flux and collimation of the X-ray source of SR, the ratio of signal to noise can be quite high, and the high sensitivity for Se measurement in Se-containing spots can be achieved by XRF. The DL for Se present on the gel in this study was about 0.20 μg g^{-1} ($N_\mathrm{b} = 23$, $N_i = 36$, $C_i = 0.5$; mean values of each ten points of the background and the standard sample), which was ideal for the quantification of Se in the Se-containing spots present on the 2-DE gel.

In addition, as described in our previous report[38] the experimental analytical precision (EAP) of XRF was represented by the standard deviation of 10 times the measurements of the net intensity of the control analyte in the same analytical context for a 95% confidence level.

$$\mathrm{EAP} = \frac{2}{m_i}\sqrt{\frac{\sum_{m=1}^{n}(I_m - \bar{I})^2}{n-1}}$$

where I_m is the m-th measurement of the net intensity of element i; and $\bar{I}$ is the mean value of the n measured values of the net intensity. The EAP enables us to estimate the random errors due to the instrument and counting statistics. The uncertainty introduced by the error source may considerably limit the accuracy and precision of the analytical results. A procedure was suggested by Rousseau[39] for evaluating the uncertainty introduced by the systematic and random errors due to the sample preparation, which mainly includes: (1) preparation of control specimens using the same preparation method as a single sample; (2) measurement of the element of interest in each of 10 specimens once using the same analytical program; (3) measurement of the element of interest in one of the specimens 10 times using the same analytical program; and (4) calculation of the relative standard deviation of both series of measurements. If the relative standard deviation obtained in

Table 1 Contents of the proteins and Se in extracted fractions from Se-enriched yeast and the Se recovery

Sample preparation	Protein content (mg)	Se extracted (μg g^{-1})	Se recovery (%)
Supernatant	20.42 ± 0.32	376.54 ± 4.28	26.68
Solid residue	—	879.83 ± 5.32	62.34
Total		1256.37	89.02[a]

[a] The total Se content in the yeast is 1411.42 ± 10.75 μg g^{-1} measured by ICP-MS.

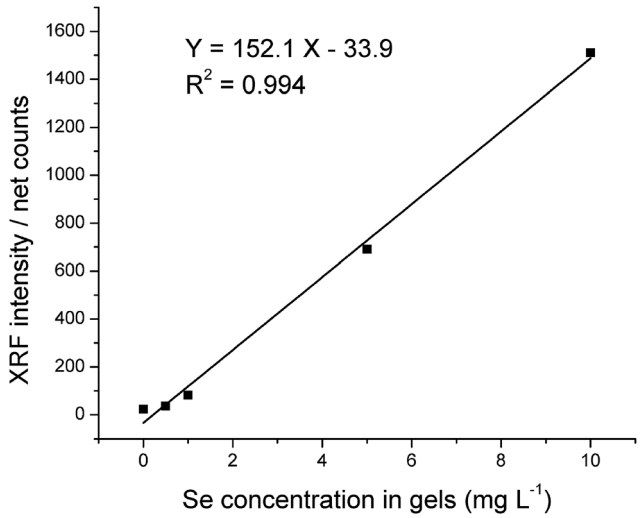

Fig. 1 Calibration curve for Se quantification using SR-XRF.

step 2 is sufficiently low, the sample preparation method is suitable. For the specific element, if the obtained relative standard deviation is quite high, the results of step 2 can be compared with those of step 3 for the purpose of determining whether the deviation is introduced by the sample preparation or by the instrument and counting statistics.

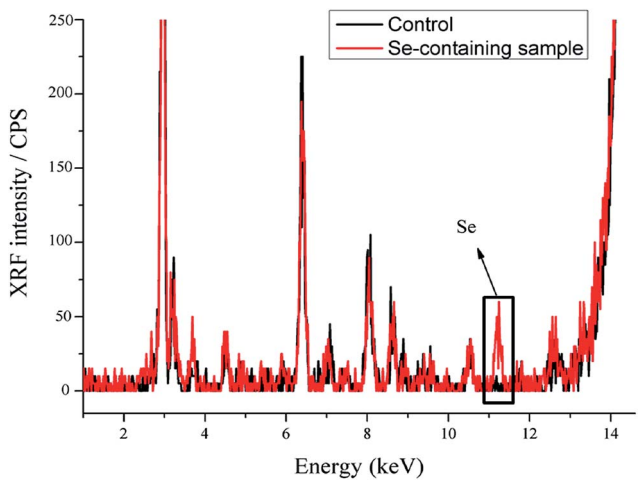

Fig. 2 The typical SR-XRF spectra of the control spot and Se-containing spot in 2-DE gels. The black line is the measurement of the control spot. The red line is the measurement of the Se-containing spot.

Protein profiles of 2-DE gel and analysis of Se in gel spots by SR-XRF

Proteins in the extract were separated using 2-DE gel electrophoresis. The protein profile of 2-DE is shown in Fig. 3a, and numerous protein spots could be visually observed on the gel.

Se in the protein spots on the 2-DE gel was identified and quantified by SR-XRF. The typical SR-XRF spectra of the control spot and the Se-containing spot in the 2-DE gels are shown in Fig. 2. Fig. 2 shows that there is a substantially strengthened Se–K peak of the Se-containing spot in the 2-DE gels compared to that of the control spot. The intensity of each point in the gel was calibrated with I_0 and Compton scattering. The patterns of Se counts of Se-containing protein spots on the 2-DE gel scanned by SR-XRF are shown in Fig. 3b. After calculation according to the DL of the XRF, only spots with a mean Se content exceeding twice the detection limit value of SR-XRF were considered to be Se-containing spots.[40,41] In this way, a number

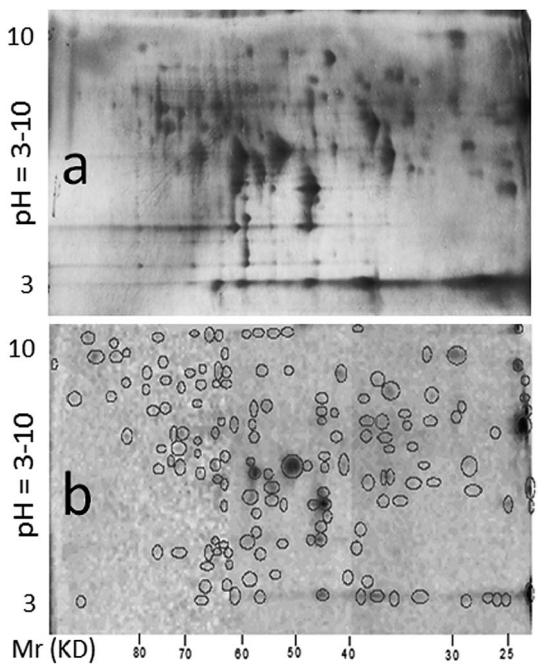

Fig. 3 Profiles of 2-D gel electrophoresis of Se-enriched yeast proteins (a); patterns of Se counts of Se-containing protein spots on 2-D gel scanned by SR-XRF (b).

Table 2 Se-containing protein spots with different molecular masses (Mr) and Se contents in Se-containing spots measured by XRF

Mr (kDa)	>80	70–80	60–70	50–60	40–50	30–40	<30	Total
Number of selenoprotein spots	11	17	34	24	19	30	20	157
Se contents in Se-containing spots (μg g^{-1})	6.71	10.49	23.22	21.13	22.97	24.43	17.61	126.56

of Se-containing protein spots and the corresponding Se contents with different molecular masses were analyzed and are shown in Table 2. About a total of 157 Se-containing protein spots in the 2-DE gel can be detected (Fig. 3b). From Table 2, 34 Se-containing protein spots with a total Se content of 23.22 μg g^{-1} and with molecular mass weights ranging from 60 kDa to 70 kDa, 30 Se-containing spots with a total Se content of 24.43 μg g^{-1} and with molecular mass weights ranging from 30–40 kDa, and 11 and 10 Se-containing protein spots with a total Se content less than 20 μg g^{-1} and with molecular masses weighing >80 kDa and <25 kDa were identified. This indicates that most of the Se-containing proteins have molecular weights between 40 and 70 kDa in Se-enriched yeast, with less in the >80 kDa and <25 kDa range. The total Se in all Se-containing proteins in the Se-enriched yeast was calculated to be 126.56 μg g^{-1} according to the calibration curve (Fig. 1). This value is lower than the total Se content of 376.54 μg g^{-1} in the extract determined by ICP-MS. This may be ascribed to the loss of water-soluble small molecular Se species during the 2-DE procedure, as well as some of the missing Se-containing proteins when analyzing the Se-containing spots on the 2-DE gel. However, the quantification of trace elements carried out by XRF combined with 2-DE can provide element content at the sub-μg g^{-1} level and high resolution in specific spots *in situ* of the electrophoresis gel with less damage to the specimen than LA-ICP-MS, which is unavailable for other common techniques.

Conclusions

The Se-containing proteins extracted from the Se-enriched yeast were successfully separated by 2-DE, and quantified by a SR-XRF technique with a high resolution and sensitivity. SR-XRF combined with 2DE is an advanced method for identifying and quantifying multi-element analysis *in situ* with a spatial resolution of several μm and sensitivity at the sub-μg g^{-1} level of elemental concentration. 2-DE coupled with SR-XRF analysis (off line) was successfully used to detect Se in Se-containing proteins in the present study. After being separated by 2-DE, Se in the protein spots was analyzed *in situ* by SR-XRF scanning with minor damage to the original samples at the sub-μg g^{-1} resolution level. In other words, based on the *in situ* imaging of specific metal-containing proteins by SR-XRF, further identification of the metal-containing proteins/peptides by electrospray ionization MS (ESI-MS) or other techniques should be more feasible in a parallel 2D gel.

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